VERSION WITH MARKINGS TO SHOW CHANGES MADE IN THE SPECIFICATION

1. The changes relative to the previous version, in page 6, line 12 to page 7, line 2, of the last paragraph are marked up as follows:

The mRNAs can be prepared from a plurality of fixed cells, wherein said fixed cells are protected from RNA degradation and also subjected to permeabilisation for enzyme penetration. Those fixed cells include fixative-treated cultural cells, frozen fresh tissues, fixative-treated fresh tissues or paraffin-embedded tissues on slides. To increase the transcriptional production of mRNAs in the step (e), the promoter sequences are preferably incorporated into the 5'-ends of said second-strand cDNAs[, including the primers of SEQ ID. 1, 3 and 5 whose annealing temperature is about 52~55°C for about 3~10 min in a consistently buffered condition (20mM Tris-HCL, pH 8.3 at 25°C, 120mM KCl, 60mM $(NH_4)_2SO_4$, 8mM $MgCl_2$, 1M betaine, 7mM DTE and 2mM spermidine) as described in Examples 1, 4 and 5 respectively]. In another aspect of this embodiment, said amplified mRNAs are preferably capped by P^1-5'-(7-methyl)-guanosine-P^3-5'-adenosine-triphosphate or P^1-5'-(7-methyl)-guanosine-P^3-5'-guanosine-triphosphate in the step (e) for further in vitro translation. On the other hand, the deoxynucleotide used in the tailing reaction of said first-strand complementary DNAs is either deoxyguanylate (dG) or deoxycytidylate (dC), and the average number of tailed nucleotides is larger than seven; most preferably, the number is about twelve. Advantageously, the final amplified mRNAs can be continuously reverse-transcribed into double-stranded cDNA by Tth-like DNA polymerase activity. The final double-stranded cDNAs are preferably cloned into competent vectors for further applications, such as transfection assay, differential screening, functional detection and so on.

2. The changes relative to the previous version, in page 8, line 6 to line 20, of the second paragraph are marked up as follows

The present invention is directed to a novel polymerase chain reaction method for mRNA amplification from single cells, named "RNA-polymerase chain reaction (RNA-PCR)". This method is primarily designed for differential screening of tissue-specific gene expressions in cell level, cloning full-length sequences of unknown gene transcripts, generating pure probes for hybridization assays, synthesizing peptides in vitro, and preparing complete cDNA libraries for gene chip technology. The purpose of the RNA-PCR relies on the repeating steps of reverse transcription, denaturation, double-stranded cDNA synthesis and in vitro transcription to bring up the population of mRNAs to two thousand folds in one cycle of above procedure. In brief, the preferred version (FIG.1) of the present invention is based on: 1) prevention of mRNA degradation [(Example 1)], 2) first reverse transcription and terminal transferase reaction to incorporate 3'-polynucleotide tails to the first-strand cDNAs [(Example 2 or 5)], 3) denaturation and then double-stranded cDNA formation based on the extension of specific promoter-primers

complementary to the 3'-polynucleotide tails [(Example 3 or 5)], 4) transcription from promoter to amplify mRNAs up to two thousand folds per round [(Example 3 or 5)], and 5) repeating aforementioned steps to achieve desired RNA amplification [(Example 5)].

3. The changes relative to the previous version of the paragraph between page 8, line 21 and page 9, line 12 are marked up as follows:

Alternatively, the second preferred version (FIG.2) of the present invention is based on: 1) prevention of mRNA degradation [(Example 1)], 2) first reverse transcription to incorporate first promoters to the 5'-ends of first-strand cDNAs and then addition of polynucleotide sequences to the 3'-ends of the first-strand cDNAs [(Example 2)], 3) double-stranded cDNA synthesis based on the extension of second promoter sequences complementary to the 3'-polynucleotide regions of the first-strand cDNAs [(Example 3)], 4) transcription to amplify either aRNAs or mRNAs up to two thousand folds in the first round of amplification cycle [(Example 3)], and 5) repeating aforementioned cycling steps to achieve desired amount of RNAs [(Example 4)]. As shown in FIG.2, the first promoter used here is different from the second promoter, resulting the control of transcription by adding different RNA polymerases. The first promoter is incorporated for aRNA amplification, whereas the second promoter is designed for mRNA amplification. By this way in conjunction with a reverse transcription step, we can choose to amplify aRNAs, first-strand cDNAs, mRNAs or second-strand cDNAs of interest, depending on which RNA polymerase and nuclease we use. Although the second and third preferred embodiments (FIGS.2 and 3) are more complicated than the first preferred embodiment (FIG.1), the principle and broad features of the second and third preferred embodiments are completely within the scope of the first preferred embodiment of the present invention.

4. The changes relative to the previous version, page 9, lines 13 to 27, of the last paragraph are marked up as follows:

As used herein, the first-strand complementary DNA (cDNA) refers to a DNA sequence which is complementary to a natural messenger RNA sequence in an A-T and C-G composition. The antisense RNA (aRNA) refers to an RNA sequence which is complementary to a natural messenger RNA sequence in an A-U and C-G composition. And, the oligo(dT)-promoter sequence refers to an RNA polymerase promoter sequence coupled with a poly-deoxythymidylate (dT) sequence in its 3'-end, of which the minimal

number of linked dT is seven; most preferably, the number is about twenty-six. The sense sequence refers to a nucleotide sequence which is in the same sequence order and composition as its homolog mRNA, whereas the antisense sequence refers to a nucleotide sequence which is complementary to its respective mRNA homologue. On the other hand, the oligo(antisense polynucleotide)-promoter sequence refers to an oligonucleotide sequence which is complementary to the polynucleotide-tail of said polynucleotide-tailed cDNAs and also linked to an RNA polymerase promoter in its 5'-end. And, the Tth-like DNA polymerases refer to RNA- and DNA-dependent DNA polymerases with reverse transcription activity[, such as AMV, M-MuLV, HIV-1 reverse transcriptases and C. therm. Polymerase].

5. The changes relative to the previous version, page 10, lines 1 to 16, of the first paragraph are marked up as follows:

By improving the methods of in-vitro transcription and in-cell RT-PCR (Embleton et.al., *Nucleic Acid Res.* (1992)), we [We] invent the [a thermal] cycling amplification of [procedure for reproducing] intracellular full-length mRNAs [(Lin et. al. *Nucleic Acid Res.* 27: 4585-4589 (1999))]. This cycling procedure preferably starts from reverse transcription of intracellular mRNAs with Tth-like DNA polymerases [(such as reverse transcription activity of C. therm. Polymerase which is initiated with primers (SEQ ID.1, 3 or 4) at about 65~72°C for about 30~60 min as described in Examples 2, 4 and 5 respectively)], following a tailing reaction with terminal transferases [(at about 37°C for about 15~20 min as described in Examples 2, 4 and 5)] and then denaturation of resulting mRNA-cDNA hybrid duplexes [(at about 94°C for about 2~3 min as described in Examples 2, 4 and 5)]. After renaturation [(at room temperature for 1 min or at about 52°C for about 3 min)] of above tailed cDNAs to specific promoter-linked primers [(SEQ ID.3 or 5)], double-stranded cDNAs are formed by Tth-like DNA polymerase[, such as C. therm. DNA polymerase activity at about 70°C for about 5 min (Examples 3 and 4) or Taq DNA polymerase activity at about 72°C for about 7 min (Example 5)]. And then, promoter-specific RNA polymerase(s)[, T7 or SP6 RNA polymerase in Example 3, 4 or 5 respectively,] is added to accomplish the transcriptional amplification of intracellular mRNAs [at about 37°C for about 1~3 hours]. The novelties of this amplification cycling procedure of the present invention are as follows: 1) single copy rare mRNAs can be increased up to 2000 folds in one round of amplification without mis-reading mistakes, 2) the mRNA amplification is linear and does not result in preferential amplification of abundant mRNA species, 3) the mRNA degradation is inhibited by fixation, and 4) the final mRNA products are of full-length and can be directly used to generate a complete cDNA library or synthesize proteins in vitro (Shi-Lung Lin et.al. *Nucleic Acid Res.* (1999)).

VERSION WITH MARKINGS TO SHOW CHANGES MADE IN THE CLAIMS

The changes relative to the previous version of the rewritten claim(s) 1, 8-10, 12, 16-17, 30, and 33 are marked up as follows.

In claim 1 (amended). A method of generating amplified messenger RNAs with polymerase reaction activity, comprising the steps of:

(a) providing a plurality of intracellular messenger RNAs for following steps (b) to (f);

(b) contacting said messenger RNAs with a plurality of first oligodeoxythymidylate-containing primers [primer sequences] to form a plurality of first-strand complementary DNAs, wherein said first-strand complementary DNAs are generated by reverse transcription of said messenger RNAs with extension of said first primers;

(c) permitting terminal extension of said first-strand complementary DNAs to form a plurality of polynucleotide-tailed first-strand complementary DNAs, wherein said polynucleotide-tailed first-strand complementary DNAs are tailed by multiple copies of deoxynucleotides;

(d) incubating denatured said polynucleotide-tailed first-strand complementary DNAs with a plurality of second [promoter-containing] primers to form a plurality of double-stranded complementary DNAs, wherein said double-stranded complementary DNAs are generated by extension of DNA polymerase activity with said second [promoter-containing] primers;

(e) permitting transcription of said double-stranded complementary DNAs to form a plurality of amplified RNAs, wherein said amplified RNAs are generated by extension of RNA polymerase activity through the promoter region of said double-stranded complementary DNAs; and

(f) contacting said amplified RNAs with said first primer sequences to form a plurality of said polynucleotide-tailed first-strand complementary DNAs, wherein said polynucleotide-tailed first-strand complementary DNAs are generated by reverse transcription of said amplified RNAs with extension of said first primer sequences.

In claim 8 (amended). The method as defined in Claim 7, wherein said enzyme activity is performed at temperature ranged from about [37°C to about 80°C] 55°C to about 75°C for Tth-like DNA polymerases with reverse transcription activity.

In claim 9 (amended). The method as defined in Claim [1] 7, wherein said first primer sequences are complementary to the tails of said messenger RNAs for the extension of reverse transcription activity in the claim 7.

In claim 10 (amended). The method as defined in Claim 9, wherein said first primer sequences are coupled to an RNA polymerase promoter and contain about eight to about thirty copies of deoxythymidylates.

In claim 12 (amended). The method as defined in Claim 1, wherein said DNA polymerase activity is an enzyme activity selected from the group consisting of E. coli DNA polymerase 1, Klenow fragment of E. coli DNA polymerase 1, T4 DNA polymerase, Taq DNA polymerase, Pwo DNA polymerase, Pfu DNA polymerase and Tth-like DNA polymerases, C. therm. Polymerase [Taq DNA polymerases and Tth-like DNA polymerases].

In claim 16 (amended). The method as defined in Claim 15, wherein said RNA polymerase promoter is selected from the group consisting of T3, T7, SP6 and M13 [T7 and SP6] RNA polymerase promoter.

In claim 17 (amended). The method as defined in Claim 1, wherein said transcription is an RNA polymerase activity selected from the group consisting of T3, T7, SP6 and M13 [T7 and SP6] RNA polymerase.

In claim 30 (amended). The method as defined in Claim 22, wherein said RNA polymerase promoter is selected from the group consisting of T3, T7, SP6 and M13 [T7 and SP6] RNA polymerase promoter.

In claim 33 (amended). The method as defined in Claim 32, wherein said mixed polymerase activities are selected from the group consisting of T3, T7, SP6, M13 [T7 and SP6] RNA polymerases and Tth-like DNA polymerases with reverse transcriptase activity, C. therm. polymerase.

REMARKS-General

1. Upon review of the original and previously amended specifications, and also in light of the observation of the Examiner noted in the above Office Action, the applicants have further amended the current specification back to the originally filed specification. No new matter has been included. The purpose of the previously amended specification was to provide a more point-to-point description of the subject matter of the instant invention, and which was abided by the antecedent basis of examples and cited references.

Response to Objection of the Specification under 35USC132

2. The applicants respectfully submit that the text added to the original pages 6, 8, 9 and 10 in the Amendment B filed May 17, 2001 have been canceled, so as to overcome the objection of the amendment B as introducing new matter into the disclosure.

Response to Rejection of Claims 1-3, 7-18, 20, 22, 23, 25, 26, and 29-35 under 35USC112

3. According to the guidance in *In re Wands,* 8 USPQ2d 1400 (CAFC 1988), the applicants respectfully submit that the original claims 1, 8-10, 12, 16, 17, 30 and 33 are amended to fully match the claimed subject matter of the instant invention to the description of the original specification and examples, as pursuant to 35USC112, first paragraph.

4. The amended claim 1 is narrowed to contain only the second preferred embodiment (FIG. 2) which was described in the contents of specification from the page 5, first line bridging to page 6, last line; from page 8, line 21 bridging to page 9, line 12; page 10, line 16-19; and the examples 1-4. As indicated in example 4, its resulting evidence is provided in figures 4a and 4b.

5. Described in detail, the claim 1(a) is described in page 6, line 8-15, and example 1 for RNA protection. The use of RNase inhibitor for the claim 1(a) is also provided in the example 2 and 4. The claim 1(b) is described in page 5, line 3-7; page 10, line 2-4, and example 2 for the step of reverse transcription. An alternative condition for claim 1(b) is further provided in example 5. The claim 1(c) is described in page 5, line 8-12; page 6, line 20-23; and page 14, line 2-6 for the tailing reaction by terminal transferase

activity. The denaturation of the resulting products of the claim 1(c) is described in page 14, line 10-12. The claim 1(d) is described in page 5, line 13-19; page 10, lines 6-7; and examples 3 and 4 for the formation of promoter-linked double-stranded DNAs as transcriptional templates. The claim 1(e) is described in page 5, line 20-23; page 9, line 2-8; page 10, line 16-19; and example 3 and 4 for transcriptional amplification of RNAs. The claim 1(f) is described in page 6, line 1-6, 9-11 and line 23-25 for the preservation of amplified RNAs. Moreover, the step-wise conjunction of claims 1(a)-1(f) is described from page 8, line 21, bridged to page 9, line 2; and examples 1-4, and shown by FIG. 2. Furthermore, the resulting evidence for the conjunction of claims 1(a)-1(f) is shown by FIGS. 4(a) and 4(b). It is clear that a detailed description of the amended claim 1 of the instant invention is provided within the meaning of 35USC112, first paragraph.

6. The claim 2 for the repeated steps (d) through (f) is described in page 6, line 7-8; and example 4 and 5. It is noted that the example 3 is the first round of transcriptional amplification while example 4 is the second round.

7. The claim 3 for intracellular RNA protection is described in page 6, line 12-15, and example 1.

8. Although the amended claims describe the practical steps and evidence of the second embodiment (FIG. 2), the other preferred embodiments (FIGS. 1 and 3) can be accomplished by the use of different first and second primers. For example, the first preferred embodiment (FIG. 1) uses a poly(dT) primer (SEQ ID. 4) as the first primer instead of a promoter-containing oligo(dT) primer (SEQ ID. 1) as shown in examples 4 and 5, while the third preferred embodiment uses the same promoter instead of different promoters. The evidence for these modifications are provided in a previously cited publication (Lin et.al. *Nucleic Acid Res.* 27: 4585-4589 (1999)). These modifications will be filed as a continuation-in-part of the present invention.

9. The claims 7 and 29 are maintained due to the highly generic similarities of these reverse transcription activities [*Oka v. Youssefyeh,* 7 USPQ2d 1169, 1171 (Fed. Cir. 1988) and *Utter v. Hiraga*, 6 USPQ2d 1709, 1714 (Fed. Cir. 1988)].

10. The claim 12 is amended due to the broadly generic similarities of these DNA polymerase activities [*Oka v. Youssefyeh,* 7 USPQ2d 1169, 1171 (Fed. Cir. 1988) and *Utter v. Hiraga*, 6 USPQ2d 1709, 1714 (Fed. Cir. 1988)].

11. The claims 16, 17, 30 and 33 are amended due to the highly generic similarities of these RNA polymerase activities [*Oka v. Youssefyeh,* 7 USPQ2d 1169, 1171 (Fed. Cir. 1988) and *Utter v. Hiraga*, 6 USPQ2d 1709, 1714 (Fed. Cir. 1988)].

12. The *Oka v. Youssefyeh,* 7 USPQ2d 1169, 1171 (Fed. Cir. 1988) suggests that the conception of a species within a genus may constitute the conception of the genus.

13. The *Utter v. Hiraga*, 6 USPQ2d 1709, 1714 (Fed. Cir. 1988) suggests that the specification may contain a written description of a broadly claimed invention without describing all species that claims encompass.

14. Regarding the guidance in *In re Wands,* 8 USPQ2d 1400 (CAFC 1988), the applicants respectfully submit that the original claims 1, 8-10, 12, 16, 17, 30 and 33 are amended to match the description of the specification of the present invention. Factors to be considered in *In re Wands,* 8 USPQ2d 1400 (CAFC 1988) are overcome by the Amendment C and in light of *Oka v. Youssefyeh,* 7 USPQ2d 1169, 1171 (Fed. Cir. 1988) and *Utter v. Hiraga*, 6 USPQ2d 1709, 1714 (Fed. Cir. 1988).

15. The quantity of experimentation necessary for the amended claims of the present invention has been provided in detail as mentioned above. The claimed invention has also been narrowed to match merely the second preferred embodiment (FIG. 2) whose practical evidence is shown by FIGS. 4(a) and 4(b). To provide sufficient experimental evidence for the first and third embodiments (FIGS. 1 and 3), some described modifications in the specification will be filed as a continuation-in-part of the present invention.

16. The amount of direction or guidance provided and the presence of working examples for the amended claims has been shown by a step-to-step description in detail as mentioned above. Such guidance points out the description and examples for each step of the amended claims of the present invention (FIG. 2), especially the claim 1(a)-1(f).

17. The nature of the present invention is related to the area of biology and chemistry, most preferably biochemistry, but not cellular physiology. Because the present invention is a combination of enzymatic reactions which are performed under an *in-vitro* (in a test tube) condition, the involvement of buffered conditions is described in detail in the examples 1-5. No workable function of the present invention is available in a living cell or by its physiology. It is understandable that an enzymatic reaction not only

involves chemical kinetics but also the biological function of the enzyme(s) used. Under many *in-vitro* conditions such as *in-vitro* transcription/translation, the use of an enzymatic reaction can successfully suppress the energetic threshold of a pure chemical reaction and, therefore, provide a feasible condition for the desired reaction(s). For a better demonstration, the original claims are amended to match the step-wise description of each enzymatic step of the present invention.

18. The state of the prior art has been developed to the point of effective reproducibility. However, none of the prior art can provide the novelties of the subject matter of the instant invention. According to the utilization of similar enzymatic reactions for different functional applications, the applicants respectfully submit a newly found prior art to support the effective reproducibility of the subject matter of the instant invention. The newly submitted prior art (US Patent No. 6,197,554 to Lin) is related to a PCR-based method for aRNA/cDNA amplification, while the present invention is based on a RNA-PCR method for mRNA amplification.

19. The relative skill of those in the art is at the same level as the referenced prior art, such as US Patent No. 5,817,465 to Mallet and No. 5,514,545 to Eberwine. A common knowledge of principal biochemistry and enzyme kinetics is required. All principal knowledge can be acquired from the referenced Sambrook's book., "*Molecular Cloning, 2nd Edition* ",Cold Spring Harbor Laboratory Press, pp8.11-8.19 (1989). Due to the fact that none of the inventors holds a Ph.D. in biochemistry, the arbitrary determination of "on par with those that hold a Ph.D. in biochemistry" is questionable.

20. The breadth of scope of the claims is narrowed to match the second preferred embodiment (FIG.2) based on a step-wise description as mentioned above. The description of RNA protection has been provided in the page 6, line 8-15; and example 1. The use of RNase inhibitor for the claim 1(a) is also provided in the example 2 and 4. On the other hand, the comparison of a primer annealing reaction to a hybridization reaction could cause misunderstanding and is misleading as pertaining to the present invention. The inventors are also experts in the field of nucleotide hybridization, and hold two US patents (US Patent No. 5,871,927 and No. 5,928,872 to Lin) in this field. Because the function of hybridization reactions (US Patent No. 5,200,313 to Carrico) is to compare the homologues between two groups of nucleic acid sequences, the stringency for hybridization reactions is much higher and sometimes different from that for primer annealing reactions. To compromise the use of promoter-containing primers in the present invention and the prior art (US Patent No. 5,514,545 to Eberwine and No.

6,197,554 to Lin), the claimed conditions are different from those of hybridization reactions for nucleotide selection. Because there is an unmatched promoter region of a promoter-containing primer, the primer annealing reactions are usually performed under a predetermined, buffered condition for permitting incomplete reassociation between partially homologous nucleotide sequences. The purpose of such partial homolog reassociation reactions is to increase the extension of nucleotide polymerization. The tested buffered conditions for the present invention have been provided in the examples 1-5.

21. The applicants believe that for all of the foregoing reasons, all of the claims 1-3, 7-18, 20, 22, 23, 25, 26, and 29-35 are in condition for allowance and such action is respectfully requested.

Respectfully submitted,

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